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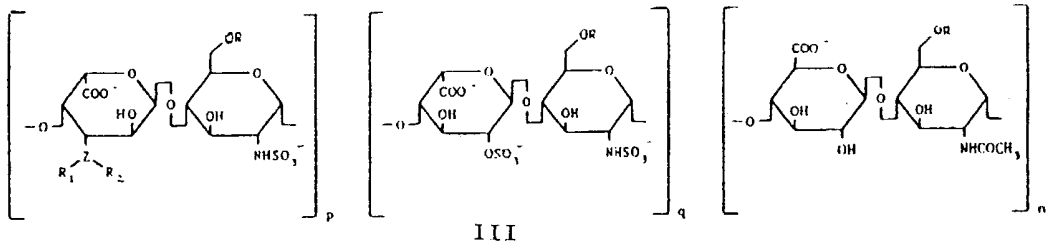
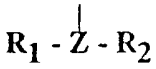
(54) Process for the synthesis of semisynthetic glycosaminoglycans containig alpha-L-galacturonic acid substituted with nucleophilic radicals in position 3.

(57) A process for the synthesis of semi-synthetic glycosaminoglycans of general formula III



III

is described in which one of the saccharide units characteristic of glycosaminoglycans with heparin or heparan structure, more specifically that of α-L-iduronic-2-O-sulfate acid, has undergone a structural modification, entirely or in part, with transformation into α-L-galacturonic acid substituted in position 3 with nucleophilic radicals of general formula II



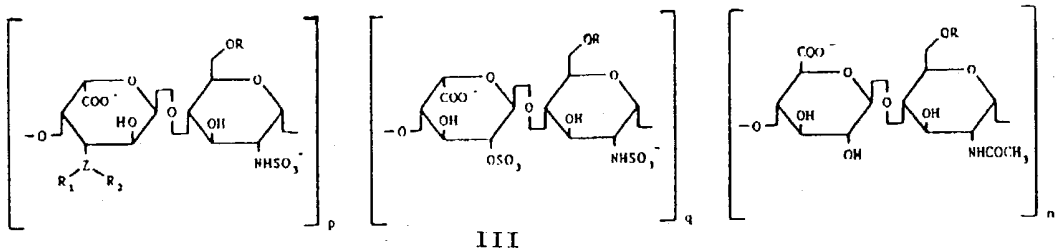
II

Said process is carried out by treating glycosaminoglycans with heparin or heparan structure by means of a nucleophilic reagent in alkaline medium.

EP 0 618 235 A1

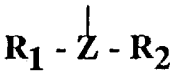
SUMMARY OF THE INVENTION

A process for the synthesis of semi-synthetic glycosaminoglycans of general formula III



III

is described in which one of the saccharide units characteristic of glycosaminoglycans with heparin or heparan structure, more specifically that of α-L-iduronic-2-O-sulfate acid, has undergone a structural modification, entirely or in part, with transformation into α-L-galacturonic acid substituted in position 3 with nucleophilic radicals of general formula II



II

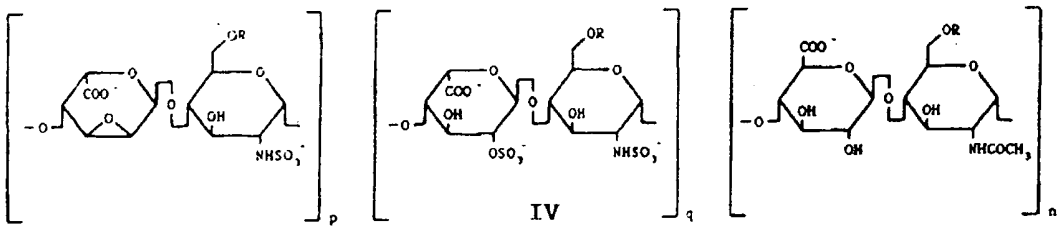
Said process is carried out by treating glycosaminoglycans with heparin or heparan structure by means of a nucleophilic reagent in alkaline medium.

BACKGROUND OF THE INVENTION

In US patent 5,010,063 a description was given of a structural modification, in basic medium, of glycosaminoglycans with heparin and heparan structure with subsequent isolation from the reaction mixture of new derivatives with respect to the state of the art, as demonstrated unmistakably by the chemical and physical characteristics and especially by the ^{13}C-NMR spectrum.

In the subsequent US patent 5,104,860 a further structural modification was described, in a basic or neutral medium, which, starting from the products formed in the reaction conditions described in US patent 5,010,063, and from the glycosaminoglycans with heparin or heparan structure used as starting products in the same patent, originated a range of new products, different from those described in said patent and new with respect to the state of the art, as demonstrated unmistakably by the chemical and physical characteristics and especially by the ^{13}C-NMR spectrum.

The chemical and physical characteristics of the products described in US patent 5,010,063 and the results of a subsequent structural study described by Jaseia M., Rej R., Sauriol F., Perlin A.S. in Can. J. Chem 67, 1449-56 (1989), with the specific aim of explaining the mechanism of the reaction of structural modification in a basic medium, have demonstrated that these derivatives show a modification which concerns just one of the saccharide units characteristic of glycosaminoglycans with heparin or heparan structure, more specifically the unit of α-L-iduronic acid sulfated in position 2 and involving its transformation into a 2,3-epoxygulonic unit. The so obtained epoxydes are represented by the following general formula IV

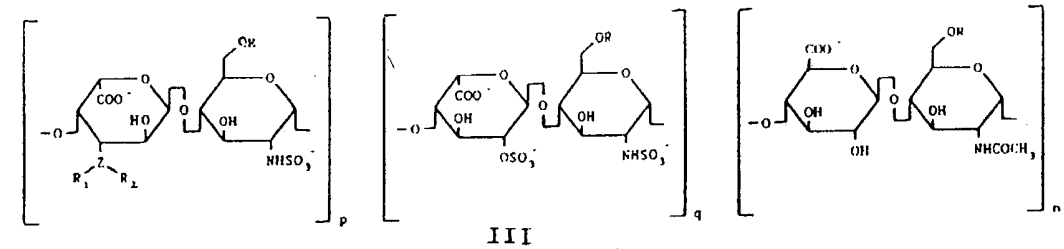


IV

Likewise it has been demonstrated that semi-synthetic glycosaminoglycans with one 2,3-epoxygulonic unit and also glycosaminoglycans with heparin or heparan structure, in conditions of reaction similar to those described in US patent 5,104,860 undergo a structural modification which also concerns the saccharide unit of α-L-iduronic acid sulfated in position 2 and involving the transformation of this saccharide unit into a unit of non-sulfated α-L-iduronic acid or α-L-galacturonic acid, according to the conditions of reaction used.

So US patent 5,010,063 describes semi-synthetic glycosaminoglycans containing an epoxy function between positions 2 and 3 of the unit of α-L-iduronic-2-O-sulfate acid taken as a starting point and the conditions of reaction necessary for obtaining them, while US patent 5,104,860 describes products deriving from further transformation of the epoxyde, confirmed as having one unit of non-sulfated α-L-iduronic or α-L-galacturonic acid, and the conditions of reaction necessary for obtaining them starting from the epoxyde itself or, as an alternative, starting from the glycosaminoglycans with heparin or heparan structure themselves, used as starting products in US patent 5,010,063.

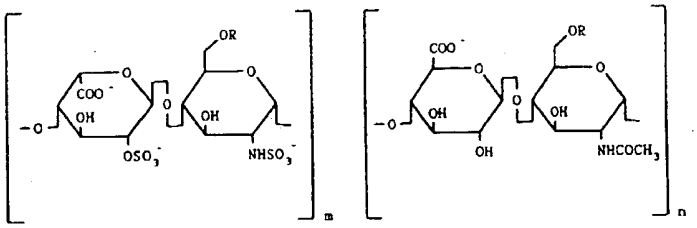
Subsequently, in published European patent application EP 565.862, semi-synthetic glycosaminoglycans were described in which one of the saccharide units characteristic of the glycosaminoglycans with heparin or heparan structure, more specifically that containing α-L-iduronic-2-O-sulfate acid, has undergone, entirely or in part, a structural modification with transformation into α-L-galacturonic acid substituted with a nucleophilic radical in position 3. The process claimed in said published European patent application describes the obtaining of the semi-synthetic glycosaminoglycans of general formula III



III

by treating the epoxydes of formula IV, described in US patent 5,010,063, with a nucleophilic reagent.

Object of the present invention is a new process for the preparation of the semi-synthetic glycosaminoglycans of general formula III directly starting from the glycosaminoglycans with heparin or heparan structure of general formula I



A B

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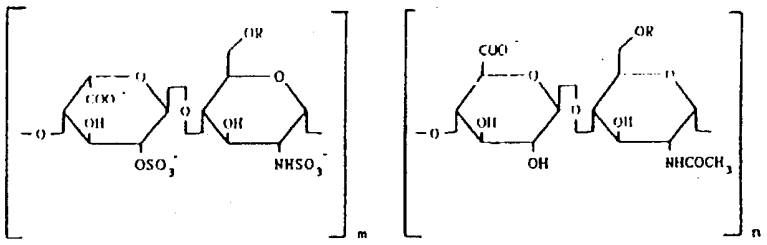
The configuration of the uronic residue different from that of the glycosaminoglycans with heparin or heparan structure was determined on the basis of the chemical physical data, particularly on the basis of the ^{13}C-NMR spectrum.

This new process represents an overcoming of the process described in the published European patent application EP 565.862 because it uses as starting product the glycosaminoglycan of formula I, while in said European patent application the starting material was the epoxy derivative of formula IV in its turn obtained by the glycosaminoglycan of formula I according to the process described in US patent 5,010,063. The advantage of directly obtaining the product of formula III in only one reaction by starting from the glycosaminoglycan of formula I instead of obtaining it by means of two consecutive reactions, the first of which includes the process of synthesis, isolation and purification of the epoxyde of formula IV starting from the glycosaminoglycan of formula I, is evident in terms of overall yield and of industrial cost.

To better define the field of the present invention, we would like to point out that the expression glycosaminoglycans with heparin or heparan structure is intended to indicate polysaccharides with a molecular weight of between about 3000 and about 50000 Daltons and characterized by the fact of possessing a disaccharide unit consisting of a uronic acid (which may be α-L-iduronic or β-D-glucuronic) and of α-D-glucosamine, connected, in alternate sequences, by 1,4-glycosidic bonds as described by Gallagher J.T. and Walker A. in Biochem. J., 230, 665-674, (1985), Lindhal U., Kjellen L. in Thrombosis and Haemostasis 66, 44-48 (1991) and by Turnbull J.E., Gallagher J.T. in Biochem. J. 273, 553-559 (1991). Since the α-L-iduronic acid can be sulfated in position 2 and the glucosamine can be N- acetylated, N-sulfated, 6-O-sulfated, 3-O-sulfated, according to the variable positions of the substituents, at least 10 different disaccharide units are possible, whose combination may generate a large number of different sequences. Bearing in mind the most represented disaccharide units and the most frequent sequences, we can say with reasonable approximation, that the general formula I can be attributed to glycosaminoglycans with heparin or heparan structure



A B

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where R represents hydrogen or the sulfate residue (SO_3^-) and where m and n are whole numbers between 1 and 100.

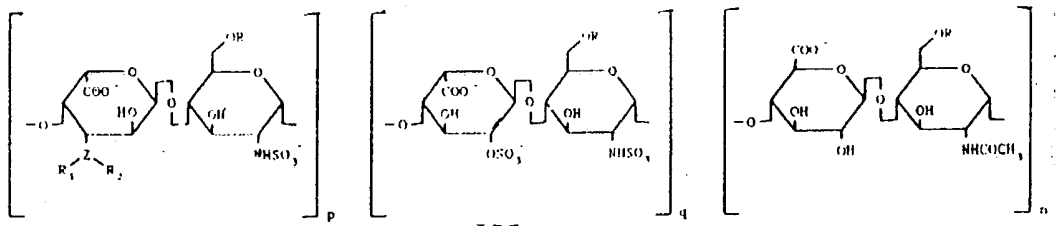
In heparin structured glycosaminoglycans of natural origin the value of m is high and the disaccharide unit A represents about 80% of the disaccharide units: on the contrary, in heparan structured glycosaminoglycans of natural origin the value of n is high and the disaccharide unit B represents about 80% of the disaccharide units.

The general formulae I and III are intended to reveal the composition of the main saccharide units but make no reference to their sequence.

As is known to experts in the art, it is possible to make a chemical modification of glycosaminoglycans of natural origin, for example through reactions of N-desulfatation, possibly followed by reactions of N-acetylation, thus also obtaining semi-synthetic N-desulfated heparins or N-desulfated-N-acetylated heparins. In addition, these glycosaminoglycans, whether natural or semi-synthetic, may be subjected to de-polymerization processes by means of which the molecular weight is taken to levels generally between 3000 and 10000 Daltons.

The structural modification described in this invention for obtaining new semi-synthetic glycosaminoglycans involves the partial or total transformation of the saccharide unit of α-L-iduronic-2-O-sulfate acid into a saccharide unit of α-L-galacturonic acid substituted by a nucleophilic radical in position 3, with the subsequent disappearance of the heparin or heparan structure. This structural modification can be done on any type of compound with heparin or heparan structure. Indeed, besides being selective, the chemical process described in this invention can be applied to glycosaminoglycans with heparin or heparan structure which present all the possible sequences; i.e. it is independent of the type and of the level of functionalization of the saccharide unit which precedes or follows in the sequence the unit of α-L-iduronic-2-O-sulfate acid which is the object of the reaction of structural modification.

The structure of the new products is represented by the general formula III



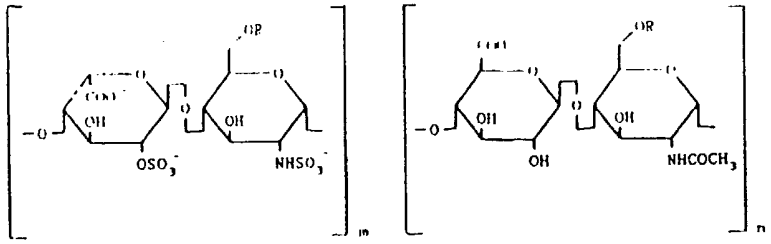
III

where p + q = m, with p other than 0, and m, n and R have the meaning as seen above, and where -Z-$(R_2)R_1$ represents the nucleophilic group introduced through the process described in this invention. The compounds obtained in this way will be indicated as "semi-synthetic glycosaminoglycans of general formula IV in which -Z$(R_2)R_1$ corresponds to ....".

The reaction of structural modification which involves the modification from saccharide unit of α-L-iduronic-2-O-sulfate acid into saccharide unit of α-L-galacturonic acid, with the introduction of the nucleophilic radical in position 3 of the α-L-galacturonic acid, does not lead to the depolymerization of the glycosaminoglycans or alteration in the distribution of the molecular weight of the polysaccharide chains which form them, and for this reason the present reaction can be applied to glycosaminoglycans with heparin or heparan structure of any molecular weight. The products obtained can however be subjected to the known processes of chemical or enzymatic depolymerization.

DETAILED DESCRIPTION OF THE INVENTION

The object of the present invention concerns a new process for obtaining semi-synthetic glycosaminoglycans in which one of the saccharide units characteristic of glycosaminoglycans with heparin or heparan structure of general formula I



A B

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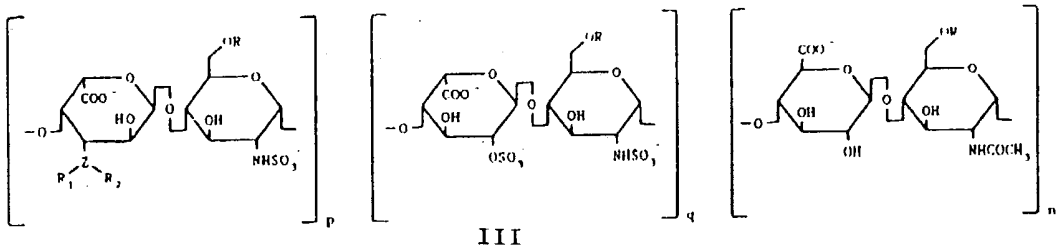
in which R represents hydrogen or the sulfate residue (SO_3^-) and m and n are whole numbers with values between 1 and 100, has undergone a structural modification with partial or total transformation of the α-L-iduronic-2-O-sulfate acid to α-L-galacturonic acid substituted in position 3 by a nucleophilic radical of general formula II

II

with formation of new semi-synthetic glycosaminoglycans of general formula III



III

where p + q = m, with p other than 0, and m, n and R have the meaning defined above.

All the nucleophilic reagents may be used to advantage in carrying out this invention and in fact the radical $-Z(R_2)R_1$ includes any type of nucleophilic reagent.

More specifically, Z represents oxygen, sulphur or nitrogen, R_1 represents the straight or branched (C_{1-12}) alkyl, aminic, aromatic, diazo or hydroxyl radicals, substituted or not substituted, and R_2 represents null or hydrogen or a straight or branched (C_{1-6}) alkyl radical, or taken with R_1 forms a heterocyclic ring.

The radicals deriving from primary or secondary amines, secondary heterocyclical amines, amino-alcohols, aminothiols, amino acids, aminoesters, peptides, alcohols, phenols, mercaptans, dithiols, thiophenols, hydroxylamines, hydrazines, hydrazides and sodium azide are preferred in performing the present invention.

Particularly preferable in performing this present invention are the radicals $-Z(R_2)R_1$ originating from the following nucleophilic reagents: glycine, glycylglycine, L-cysteine, acetyl-L-cysteine, L-cysteine ethyl ester, 2-aminothiophenol, 1,3-propandithiol, cysteamine, sodium azide, 2-aminoethyl bisulfate, taurine, thioglycolic acid, β-alanine ethyl ester, L-cystine, hydroxylamine, glycyltaurine, cysteinyltaurine, glycylcysteine, glycyl-phenylalanine, glycyltyrosine, 2-aminoethanol, glycine ester with 2-aminoethanol, glycine amide with 2-aminoethanol, arginyllysine, arginine, lysine, 2-aminoethanol ester with acetic acid, salicylic acid, methionine, glycylproline, γ-aminobutyric acid, lysylprolylarginine, threonyllysylproline, threonyllysine, prolylarginine, lysylproline, choline, 4-(3-aminopropyl)-2-hydroxybenzoic acid and 4-(2-aminoethyl)-2-hydroxybenzoic acid.

The process for obtaining semi-synthetic glycosaminoglycans of general formula III involves reacting a glycosaminoglycan with heparin or heparan structure of general formula I with a nucleophilic reagent whose radical is included in the general formula II, in aqueous solution and in the presence of a quantity of inorganic or organic base able to salify any acid groups present in the nucleophilic reagents and/or to free the same nucleophilic reagents from any salts they may have with substances of an acid nature and to generate such an excess of alkalinity that the reaction mixture is between 0.5 and 6 N as regards the base used, preferably from 1 to 3N. The reaction is done by adding the glycosaminoglycan of formula I, in a quantity comprised between 1 % and 5% with respect to the end volume of the solution, to an aqueous solution containing the nucleophilic reagent and the inorganic or organic base; the same nucleophilic reagent can act as a base when it is a strong base.

The quantity of nucleophilic agent is comprised between 1 and 200 molar equivalents, preferably between 10 and 100 molar equivalents, with respect to the dimeric unit of the glycosaminoglycan of formula I. Alkaline or alkaline-earth hydroxides, preferably sodium or potassium hydroxide, are used as inorganic bases, while tertiary amines like triethylamine are the organic bases preferably used. The reaction mixture is kept under stirring, possibly in an atmosphere of inert gases, preferably nitrogen, where the nucleophilic reagent is easily oxidizable, at a temperature of between 45 °C and 95 °C, preferably between 50 °C and 70 °C, for a period of time of between 30 minutes and 24 hours, preferably between 2 and 6 hours.

At the end of the reaction, after cooling, the reaction mixture is given a neutral pH by adding an aqueous solution of hydrochloric acid. The excess of nucleophilic reagent may optionally be removed, for example through extraction with a solvent which is not miscible with water, with chloroform or diethyl ether, or through filtration where it is not soluble in aqueous medium with neutral pH. The clear aqueous solution may be further purified at a later stage through dialysis, cut off 3000 Daltons, first in running water and then in distilled water. Finally the semi-synthetic glycosaminoglycan of general formula III is isolated through lyophilization of the aqueous solution which contains it or through precipitation on addition of a suitable solvent.

The examples below are a further illustration of the invention but they must not be taken as a limitation of the invention itself.

EXAMPLE 1

Semi-synthetic glycosaminoglycan of general formula III in which $-Z(R_2)R_1$ corresponds to glycyl.

400 Milligrams of heparin sodium salt are added to 20 ml of an aqueous solution containing 4500 mg of glycine and 4000 mg of sodium hydroxide, thermostated at 60 °C. The reaction mixture is kept under stirring at 60 °C for 3 hours, is then cooled to room temperature and the pH is neutralized through the addition of a diluted aqueous solution of hydrochloric acid. The solution is then subjected to dialysis, cut off 3000 Daltons, for 12 hours in running water and for 6 hours in distilled water and is finally lyophilized obtaining 380 mg of product

EXAMPLE 2

Semi-synthetic glycosaminoglycan of general formula III in which $-Z(R_2)R_1$ corresponds to taurinyl.

The reaction is performed in the same conditions as described in example 1 using 3750 mg of taurine instead of 4500 mg of glycine and obtaining 400 mg of product.

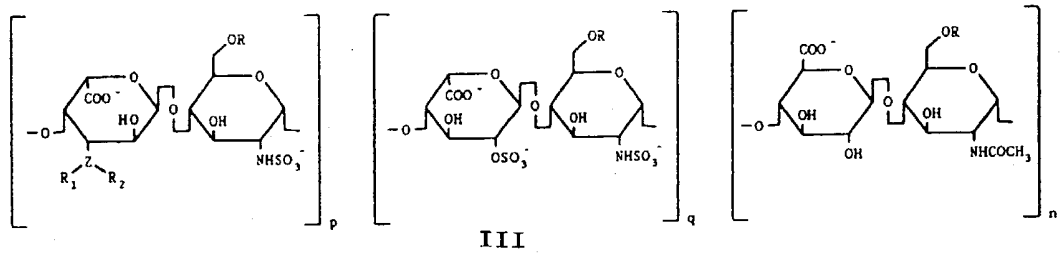
EXAMPLE 3

Semi-synthetic glycosaminoglycan of general formula III in which $-Z(R_2)R_1$ corresponds to 1-amino-3-carboxypropane.

The reaction is performed in the same conditions as described in example 1 using 6200 mg of 4-aminobutanoic acid instead of 4500 mg of glycine and 3200 mg of sodium hydroxide instead of 4000 mg and extending the time of reaction to 4 hours. 390 Mg of product are obtained.

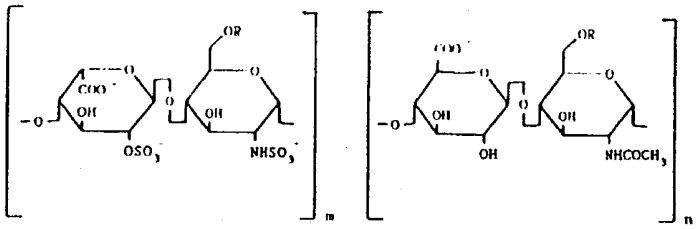
Claims

1. Process for the synthesis of semi-synthetic glycosaminoglycans of general formula III



III

where p + q = m, with p other than 0, and m and n are whole numbers whose value is between 1 and 100, R represents hydrogen or the sulfate residue (SO_3^-) and $-Z(R_2)R_1$ represents a nucleophilic radical, which includes reacting a glycosaminoglycan with heparin or heparan structure of general formula I



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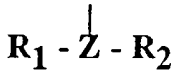
with from 1 to 200 molar equivalents, with respect to the dimeric unit of the glycosaminoglycan with heparin or heparan structure of general formula I, of a nucleophilic reagent whose radical is included within the general formula II



II

in an aqueous solution containing a quantity of inorganic or organic base sufficient to salify any acid groups present in the nucleophilic reagents and/or to release the same nucleophilic reagents from any salts they may have with substances of an acid nature and to generate an excess of alkalinity such that the reaction mixture is from 0.5 to 6 N with respect to the base used, optionally in an atmosphere of inert gas, under stirring for a period of time between 30 minutes and 24 hours at a temperature between 45 °C and 95 °C, neutralizing the pH of the cold aqueous solution through addition of an aqueous solution of hydrochloric acid, optionally removing the excess of nucleophilic reagent through extraction with a solvent not miscible with water or through filtration, subjecting the aqueous solution to dialysis with running water and with distilled water and isolating the product by means of lyophilization of the aqueous solution containing it or through precipitation by addition of a suitable solvent.

2. Process according to claim 1 characterized by the fact that the quantity of nucleophilic reagent is between 10 and 100 molar equivalents with respect to the dimeric unit of the glycosaminoglycan of general formula I and that the concentration of said glycosaminoglycan in aqueous solution is between

1% and 5%.

3. Process according to claim 1 characterized by the fact that the base used is selected from sodium hydroxide, potassium hydroxide and triethylamine and that the excess of alkalinity is such that the reaction mixture becomes from 1N to 3N with respect to the base used.

4. Process according to each of the previous claims characterized by the fact that Z represents oxygen, sulphur, or nitrogen, R_1 represents the straight or branched (C_{1-12}) alkyl, aminic, aromatic, diazoic or hydroxyl radicals, substituted or not substituted and R_2 represents null or a straight or branched (C_{1-6}) alkyl radical, or taken with R_1 forms a heterocyclic ring.

5. Process according to claim 4 characterized by the fact that the radical $-Z(R_2)R_1$ derives from primary or secondary amines, secondary heterocyclic amines, aminoalcohols, aminothiols, aminoacids, aminoesters, peptides, alcohols, phenols, mercaptans, dithiols, thiophenols, hydroxylamines, hydrazines, hydrazides and sodium azide.

6. Process according to claim 5 characterized by the fact that the radical $-Z(R_2)R_1$ derives from glycine, glycylglycine, L-cysteine, acetyl-L-cysteine, L-cysteine ethyl ester, 2-aminothiophenol, 1,3-propandithiol, cysteamine, sodium azide, 2-aminoethyl bisulfate, taurine, thioglycolic acid, β-alanine ethyl ester, L-cystine, hydroxylamine, glycyltaurine, cysteinyltaurine, glycylcysteine, glycylphenylalanine, glycyltyrosine, 2-aminoethanol, glycine ester with 2-aminoethanol, glycine amide with 2-aminoethanol, arginyllysine, arginine, lysine, 2-aminoethanol ester with acetic acid, salicylic acid, methionine, glycylproline, γ-aminobutyric acid, lysylprolylarginine, threonyllysylproline, threonyllysine, prolylarginine, lysylproline, choline, 4-(3-aminopropyl)-2-hydroxybenzoic acid and 4-(2-aminoethyl)-2-hydroxybenzoic acid.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 10 4309

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
D,A	US-A-5 010 063 (PIANI ET AL.)		C08B37/10
A	CARBOHYDRATE RESEARCH vol. 200 , 1990 , AMSTERDAM pages 437 - 447 RABINDRA N. REJ ET AL. 'Base-catalyzed conversion of the alpha-L-iduronic acid 2-sulfate unit of heparin into a unit of alpha-L-galacturonic acid, and related reactions.'		
A	EP-A-0 485 748 (ALFA WASSERMANN)		
D,P,X	EP-A-0 565 862 (ALFA WASSERMANN) 20 October 1993 *The whole document*	1-6	

TECHNICAL FIELDS SEARCHED (Int.Cl.5)
C08B C07H

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	30 June 1994	Lensen, H

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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EPO FORM 1503 03.82 (P04C04)

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(21) Application number: **94104309.3**

(22) Date of filing: **18.03.1994**

(54) Process for the synthesis of semisynthetic glycosaminoglycans containing alpha-L-galacturonic acid substituted with nucleophilic radicals in position 3

Verfahren zur Herstellung von halbsynthetischen Glykosaminoglykanen, die in 3-Position mit nukleophilen Radikalen substituierte alpha-L-Galakturonsäure enthalten

Procédé pour la préparation de glycosaminoglycanes semi-synthétiques contenant l'acide alpha L-galacturonique substitué en position 3 par des radicaux nucléophiles

(84) Designated Contracting States:
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EP-A- 0 485 748 **EP-A- 0 565 862**
US-A- 5 010 063

- **CARBOHYDRATE RESEARCH vol. 200 , 1990 , AMSTERDAM pages 437 - 447 RABINDRA N. REJ ET AL. 'Base-catalyzed conversion of the alpha-L-iduronic acid 2-sulfate unit of heparin into a unit of alpha-L-galacturonic acid, and related reactions.'**

EP 0 618 235 B1

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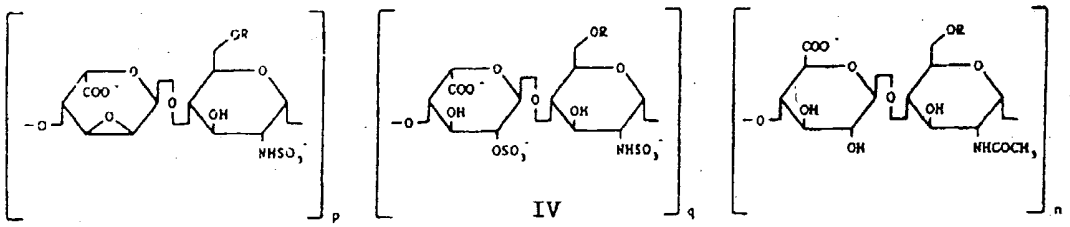
Description

BACKGROUND OF THE INVENTION

In US patent 5,010,063 a description was given of a structural modification, in basic medium, of glycosaminoglycans with heparin and heparan structure with subsequent isolation from the reaction mixture of new derivatives with respect to the state of the art, as demonstrated unmistakably by the chemical and physical characteristics and especially by the ^{13}C-NMR spectrum.

In the subsequent US patent 5,104,860 a further structural modification was described, in a basic or neutral medium, which, starting from the products formed in the reaction conditions described in US patent 5,010,063, and from the glycosaminoglycans with heparin or heparan structure used as starting products in the same patent, originated a range of new products, different from those described in said patent and new with respect to the state of the art, as demonstrated unmistakably by the chemical and physical characteristics and especially by the ^{13}C-NMR spectrum.

The chemical and physical characteristics of the products described in US patent 5,010,063 and the results of a subsequent structural study described by Jaseia M., Rej R., Sauriol F., Perlin A.S. in Can. J. Chem 67, 1449-56 (1989), with the specific aim of explaining the mechanism of the reaction of structural modification in a basic medium, have demonstrated that these derivatives show a modification which concerns just one of the saccharide units characteristic of glycosaminoglycans with heparin or heparan structure, more specifically the unit of α-L-iduronic acid sulfated in position 2 and involving its transformation into a 2,3-epoxygulonic unit. The so obtained epoxydes are represented by the following general formula IV

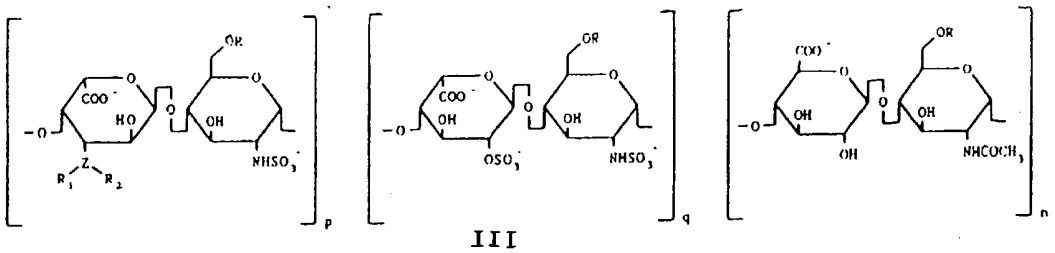


IV

Likewise it has been demonstrated that semi-synthetic glycosaminoglycans with one 2,3-epoxygulonic unit and also glycosaminoglycans with heparin or heparan structure, in conditions of reaction similar to those described in US patent 5,104,860 undergo a structural modification which also concerns the saccharide unit of α-L-iduronic acid sulfated in position 2 and involving the transformation of this saccharide unit into a unit of non-sulfated α-L-iduronic acid or α-L-galacturonic acid, according to the conditions of reaction used.

So US patent 5,010,063 describes semi-synthetic glycosaminoglycans containing an epoxy function between positions 2 and 3 of the unit of α-L-iduronic-2-O-sulfate acid taken as a starting point and the conditions of reaction necessary for obtaining them, while US patent 5,104,860 describes products deriving from further transformation of the epoxyde, confirmed as having one unit of non-sulfated α-L-iduronic or α-L-galacturonic acid, and the conditions of reaction necessary for obtaining them starting from the epoxyde itself or, as an alternative, starting from the glycosaminoglycans with heparin or heparan structure themselves, used as starting products in US patent 5,010,063.

Subsequently, in published European patent application EP 565.862, semi-synthetic glycosaminoglycans were described in which one of the saccharide units characteristic of the glycosaminoglycans with heparin or heparan structure, more specifically that containing α-L-iduronic-2-O-sulfate acid, has undergone, entirely or in part, a structural modification with transformation into α-L-galacturonic acid substituted with a nucleophilic radical in position 3. The process claimed in said published European patent application describes the obtaining of the semi-synthetic glycosaminoglycans of general formula III

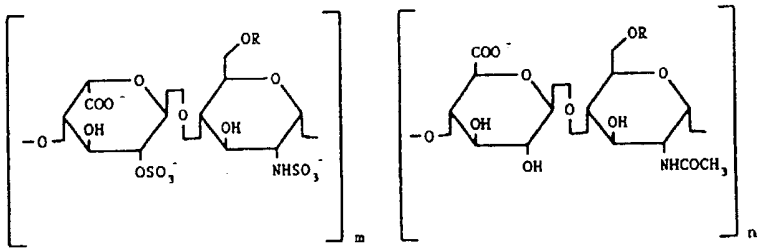


III

by treating the epoxydes of formula IV, described in US patent 5,010,063, with a nucleophilic reagent.

Carbohydrate Research, Vol. 200 (1990), pages 437-447 relates to the discovery that residues of α-L-idopyranosyluronic acid 2-sulfate in heparin undergo a loss of the sulfate group by intramolecular displacement in sodium carbonate solution at 100°, leading to the formation of a heparin analog containing residues of α-L-galactopyranosyluronic acid. This formation is due to the selective hydrolysis of the oxirane ring of 2,3-anhydro-α-L-guluronic acid, a transient intermediate formed during the desulfation step. Hence, this document teaches that aqueous solutions containing nucleophilic reagents react with 2,3-anhydro-α-L-guluronic acid to give the hydrolysis product, i.e. α-L-galactopyranosyluronic acid, while no other nucleophilic group reacts with the 2,3-epoxide intermediate.

Object of the present invention is a new process for the preparation of the semi-synthetic glycosaminoglycans of general formula III directly starting from the glycosaminoglycans with heparin or heparan structure of general formula I



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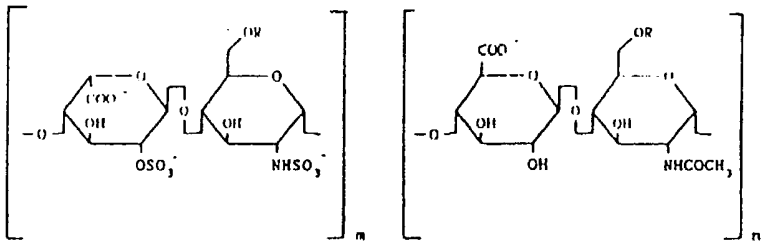
The configuration of the uronic residue different from that of the glycosaminoglycans with heparin or heparan structure was determined on the basis of the chemical physical data, particularly on the basis of the ^{13}C-NMR spectrum.

This new process represents an overcoming of the process described in the published European patent application EP 565.862 because it uses as starting product the glycosaminoglycan of formula I, while in said European patent application the starting material was the epoxy derivative of formula IV in its turn obtained by the glycosaminoglycan of formula I according to the process described in US patent 5,010,063. The advantage of directly obtaining the product of formula III in only one reaction by starting from the glycosaminoglycan of formula I instead of obtaining it by means of two consecutive reactions, the first of which includes the process of synthesis, isolation and purification of the epoxyde of formula IV starting from the glycosaminoglycan of formula I, is evident in terms of overall yield and of industrial cost.

To better define the field of the present invention, we would like to point out that the expression glycosaminoglycans with heparin or heparan structure is intended to indicate polysaccharides with a molecular weight of between about 3000 and about 50000 Daltons and characterized by the fact of possessing a disaccharide unit consisting of a uronic acid (which may be α-L-iduronic or β-D-glucuronic) and of α-D-glucosamine, connected, in alternate sequences, by 1,4-glycosidic bonds as described by Gallagher J.T. and Walker A. in Biochem. J., 230, 665-674, (1985), Lindhal U., Kjellen L. in Thrombosis and Haemostasis 66, 44-48 (1991) and by Turnbull J.E., Gallagher J.T. in Biochem. J. 273, 553-559 (1991). Since the α-L-iduronic acid can be sulfated in position 2 and the glucosamine can be N- acetylated, N-sulfated, 6-O-sulfated, 3-O-sulfated, according to the variable positions of the substituents, at least 10 different disaccharide units are possible, whose combination may generate a large number of different sequences. Bearing in mind the most represented disaccharide units and the most frequent sequences, we can say with reasonable approximation, that the general formula I can be attributed to glycosaminoglycans with heparin or heparan structure



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where R represents hydrogen or the sulfate residue (SO_3^-) and where m and n are whole numbers between 1 and 100.

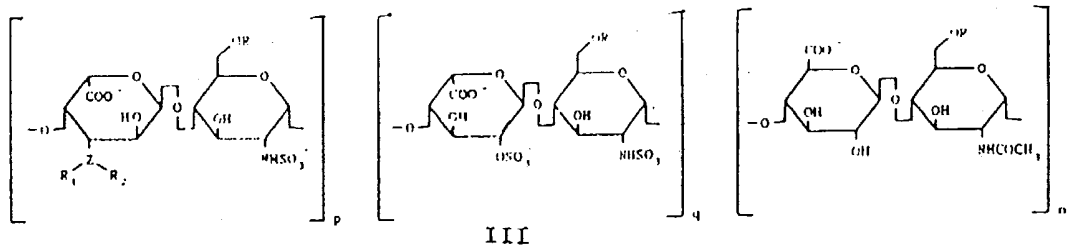
In heparin structured glycosaminoglycans of natural origin the value of m is high and the disaccharide unit A represents about 80% of the disaccharide units: on the contrary, in heparan structured glycosaminoglycans of natural origin the value of n is high and the disaccharide unit B represents about 80% of the disaccharide units.

The general formulae I and III are intended to reveal the composition of the main saccharide units but make no reference to their sequence.

As is known to experts in the art, it is possible to make a chemical modification of glycosaminoglycans of natural origin, for example through reactions of N-desulfatation, possibly followed by reactions of N-acetylation, thus also obtaining semi-synthetic N-desulfated heparins or N-desulfated-N-acetylated heparins. In addition, these glycosaminoglycans, whether natural or semi-synthetic, may be subjected to depolymerization processes by means of which the molecular weight is taken to levels generally between 3000 and 10000 Daltons.

The structural modification described in this invention for obtaining new semi-synthetic glycosaminoglycans involves the partial or total transformation of the saccharide unit of α-L-iduronic-2-O-sulfate acid into a saccharide unit of α-L-galacturonic acid substituted by a nucleophilic radical in position 3, with the subsequent disappearance of the heparin or heparan structure. This structural modification can be done on any type of compound with heparin or heparan structure. Indeed, besides being selective, the chemical process described in this invention can be applied to glycosaminoglycans with heparin or heparan structure which present all the possible sequences; i.e. it is independent of the type and of the level of functionalization of the saccharide unit which precedes or follows in the sequence the unit of α-L-iduronic-2-O-sulfate acid which is the object of the reaction of structural modification.

The structure of the new products is represented by the general formula III



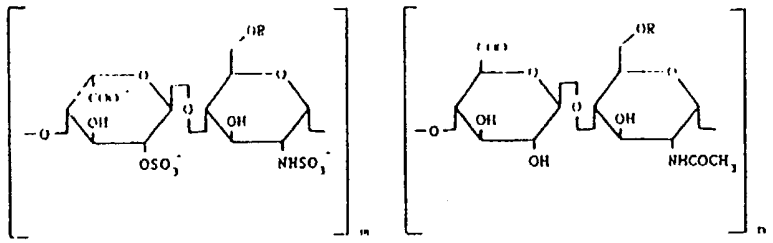
III

where p + q = m , with p other than 0, and m, n and R have the meaning as seen above, and where $-Z(R_2)R_1$ represents the nucleophilic group introduced through the process described in this invention. The compounds obtained in this way will be indicated as "semi-synthetic glycosaminoglycans of general formula IV in which $-Z(R_2)R_1$ corresponds to ....".

The reaction of structural modification which involves the modification from saccharide unit of α-L-iduronic-2-O-sulfate acid into saccharide unit of α-L-galacturonic acid, with the introduction of the nucleophilic radical in position 3 of the α-L-galacturonic acid, does not lead to the depolymerization of the glycosaminoglycans or alteration in the distribution of the molecular weight of the polysaccharide chains which form them, and for this reason the present reaction can be applied to glycosaminoglycans with heparin or heparan structure of any molecular weight. The products obtained can however be subjected to the known processes of chemical or enzymatic depolymerization.

DETAILED DESCRIPTION OF THE INVENTION

The object of the present invention concerns a new process for obtaining semi-synthetic glycosaminoglycans in which one of the saccharide units characteristic of glycosaminoglycans with heparin or heparan structure of general formula I



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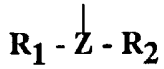
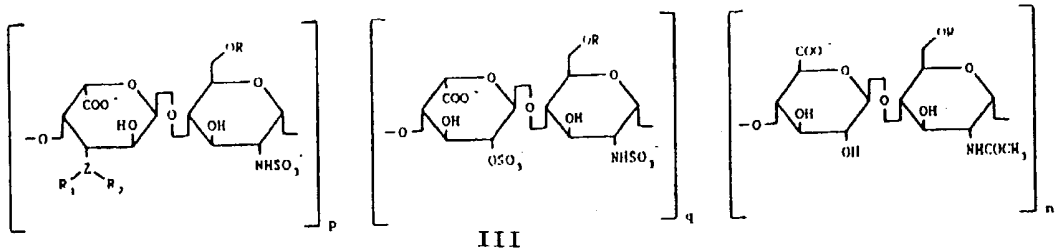
in which R represents hydrogen or the sulfate residue (SO_3^-) and m and n are whole numbers with values between 1 and 100, has undergone a structural modification with partial or total transformation of the α-L-iduronic-2-O-sulfate acid to α-L-galacturonic acid substituted in position 3 by a nucleophilic radical of general formula II



II

with formation of new semi-synthetic glycosaminoglycans of general formula III



III

where p + q = m , with p other than 0, and m, n and R have the meaning defined above and

Z represents oxygen, sulphur or nitrogen, R_1 represents the straight or branched (C_{1-12}) alkyl, aminic, aromatic, diazo or hydroxyl radicals, substituted or not substituted, and R_2 represents null or hydrogen or a straight or branched (C_{1-6}) alkyl radical, or taken with R_1 forms a heterocyclic ring.

The radicals deriving from primary or secondary amines, secondary heterocyclical amines, amino-alcohols, aminothiols, amino acids, aminoesters, peptides, alcohols, phenols, mercaptans, dithiols, thiophenols, hydroxylamines, hydrazines, hydrazides and sodium azide are preferred in performing the present invention.

Particularly preferable in performing this present invention are the radicals $-Z(R_2)R_1$ originating from the following nucleophilic reagents: glycine, glycylglycine, L-cysteine, acetyl-L-cysteine, L-cysteine ethyl ester, 2-aminothiophenol, 1,3-propandithiol, cysteamine, sodium azide, 2-aminoethyl bisulfate, taurine, thioglycolic acid, β-alanine ethyl ester, L-cystine, hydroxylamine, glycyltaurine, cysteinyltaurine, glycylcysteine, glycylphenylalanine, glycyltyrosine, 2-aminoethanol, glycine ester with 2-aminoethanol, glycine amide with 2-aminoethanol, arginyllysine, arginine, lysine, 2-aminoethanol ester with acetic acid, salicylic acid, methionine, glycylproline, γ-aminobutyric acid, lysylprolylarginine, threonyllysylproline, threonyllysine, prolylarginine, lysylproline, choline, 4-(3-aminopropyl)-2-hydroxybenzoic acid and 4-(2-aminoethyl)-2-hydroxybenzoic acid.

The process for obtaining semi-synthetic glycosaminoglycans of general formula III involves reacting a gly-

cosaminoglycan with heparin or heparan structure of general formula I with a nucleophilic reagent whose radical is included in the general formula II, in aqueous solution and in the presence of a quantity of inorganic or organic base able to salify any acid groups present in the nucleophilic reagents and/or to free the same nucleophilic reagents from any salts they may have with substances of an acid nature and to generate such an excess of alkalinity that the reaction mixture is between 0.5 and 6 N as regards the base used, preferably from 1 to 3N. The reaction is done by adding the glycosaminoglycan of formula I, in a quantity comprised between 1 % and 5% with respect to the end volume of the solution, to an aqueous solution containing the nucleophilic reagent and the inorganic or organic base; the same nucleophilic reagent can act as a base when it is a strong base.

The quantity of nucleophilic agent is comprised between 1 and 200 molar equivalents, preferably between 10 and 100 molar equivalents, with respect to the dimeric unit of the glycosaminoglycan of formula I. Alkaline or alkaline-earth hydroxides, preferably sodium or potassium hydroxide, are used as inorganic bases, while tertiary amines like triethylamine are the organic bases preferably used. The reaction mixture is kept under stirring, possibly in an atmosphere of inert gases, preferably nitrogen, where the nucleophilic reagent is easily oxidizable, at a temperature of between 45°C and 95°C, preferably between 50°C and 70°C, for a period of time of between 30 minutes and 24 hours, preferably between 2 and 6 hours.

At the end of the reaction, after cooling, the reaction mixture is given a neutral pH by adding an aqueous solution of hydrochloric acid. The excess of nucleophilic reagent may optionally be removed, for example through extraction with a solvent which is not miscible with water, with chloroform or diethyl ether, or through filtration where it is not soluble in aqueous medium with neutral pH. The clear aqueous solution may be further purified at a later stage through dialysis, cut off 3000 Daltons, first in running water and then in distilled water. Finally the semi-synthetic glycosaminoglycan of general formula III is isolated through lyophilization of the aqueous solution which contains it or through precipitation on addition of a suitable solvent.

The examples below are a further illustration of the invention but they must not be taken as a limitation of the invention itself.

EXAMPLE 1

Semi-synthetic glycosaminoglycan of general formula III in which $-Z(R_2)R_1$ corresponds to glycyl.

400 Milligrams of heparin sodium salt are added to 20 ml of an aqueous solution containing 4500 mg of glycine and 4000 mg of sodium hydroxide, thermostated at 60°C. The reaction mixture is kept under stirring at 60°C for 3 hours, is then cooled to room temperature and the pH is neutralized through the addition of a diluted aqueous solution of hydrochloric acid. The solution is then subjected to dialysis, cut off 3000 Daltons, for 12 hours in running water and for 6 hours in distilled water and is finally lyophilized obtaining 380 mg of product

EXAMPLE 2

Semi-synthetic glycosaminoglycan of general formula III in which $-Z(R_2)R_1$ corresponds to taurinyl.

The reaction is performed in the same conditions as described in example 1 using 3750 mg of taurine instead of 4500 mg of glycine and obtaining 400 mg of product.

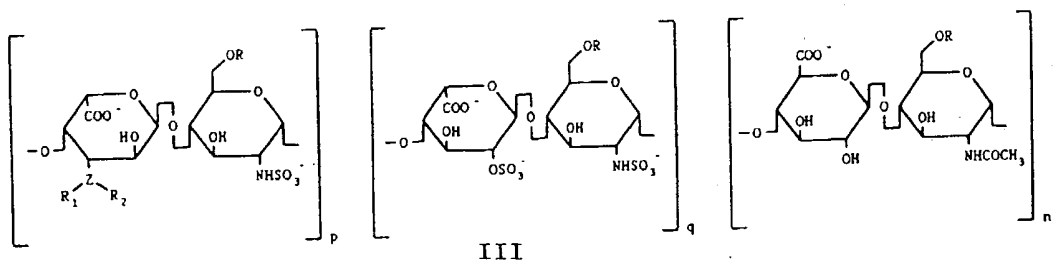
EXAMPLE 3

Semi-synthetic glycosaminoglycan of general formula III in which $-Z(R_2)R_1$ corresponds to 1-amino-3-carboxy-propane.

The reaction is performed in the same conditions as described in example 1 using 6200 mg of 4-aminobutanoic acid instead of 4500 mg of glycine and 3200 mg of sodium hydroxide instead of 4000 mg and extending the time of reaction to 4 hours. 390 Mg of product are obtained.

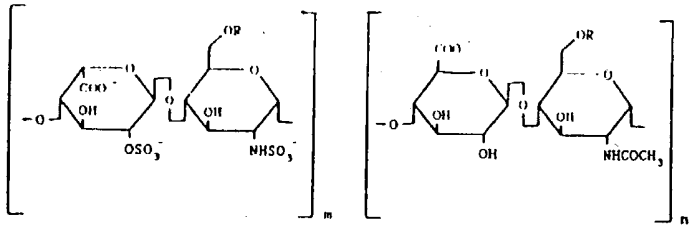
Claims

1. Process for the synthesis of semi-synthetic glycosaminoglycans of general formula III



III

where p + q = m , with p other than 0, and m and n are whole numbers whose value is between 1 and 100, R represents hydrogen or the sulfate residue (SO_3^-) and $-Z(R_2)R_1$ represents a nucleophilic radical, in which Z represents oxygen, sulphur, or nitrogen, R_1 represents the straight or branched (c_{1-12}) alkyl, aminic, aromatic, diazoic or hydroxyl radicals, substituted or not substituted and R_2 represents null or a straight or branched (c_{1-6}) alkyl radical, or taken with R_1 forms a heterocyclic ring, which includes reacting a glycosaminoglycan with heparin or heparan structure of general formula I



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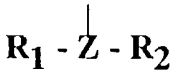
with from 1 to 200 molar equivalents, with respect to the dimeric unit of the glycosaminoglycan with heparin or heparan structure of general formula I, of a nucleophilic reagent whose radical is included within the general formula II



II

in an aqueous solution containing a quantity of inorganic or organic base sufficient to salify any acid groups present in the nucleophilic reagents and/or to release the same nucleophilic reagents from any salts they may have with substances of an acid nature and to generate an excess of alkalinity such that the reaction mixture is from 0.5 to 6 N with respect to the base used, optionally in an atmosphere of inert gas, under stirring for a period of time between 30 minutes and 24 hours at a temperature between 45°C and 95°C, neutralizing the pH of the cold aqueous solution through addition of an aqueous solution of hydrochloric acid, optionally removing the excess of nucleophilic reagent through extraction with a solvent not miscible with water or through filtration, subjecting the aqueous solution to dialysis with running water and with distilled water and isolating the product by means of lyophilization of the aqueous solution containing it or through precipitation by addition of a suitable solvent.

2. Process according to claim 1 characterized by the fact that the quantity of nucleophilic reagent is between 10 and 100 molar equivalents with respect to the dimeric unit of the glycosaminoglycan of general formula I and that the concentration of said glycosaminoglycan in aqueous solution is between 1% and 5%.

3. Process according to claim 1 characterized by the fact that the base used is selected from sodium hydroxide, potassium hydroxide and triethylamine and that the excess of alkalinity is such that the reaction mixture becomes from

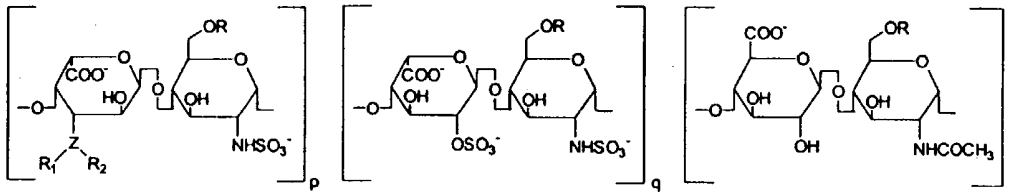
1N to 3N with respect to the base used.

4. Process according to claim 1 characterized by the fact that the radical $-Z(R_2)R_1$ derives from primary or secondary amines, secondary heterocyclic amines, aminoalcohols, aminothiols, aminoacids, aminoesters, peptides, alcohols, phenols, mercaptans, dithiols, thiophenols, hydroxylamines, hydrazines, hydrazides and sodium azide.

5. Process according to claim 4 characterized by the fact that the radical $-Z(R_2)R_1$ derives from glycine, glycylglycine, L-cysteine, acetyl-L-cysteine, L-cysteine ethyl ester, 2-aminothiophenol, 1,3-propandithiol, cysteamine, sodium azide, 2-aminoethyl bisulfate, taurine, thioglycolic acid, β-alanine ethyl ester, L-cystine, hydroxylamine, glycyltaurine, cysteinyltaurine, glycylcysteine, glycylphenylalanine, glycyltyrosine, 2-aminoethanol, glycine ester with 2-aminoethanol, glycine amide with 2-aminoethanol, arginyllysine, arginine, lysine, 2-aminoethanol ester with acetic acid, salicylic acid, methionine, glycylproline, γ-aminobutyric acid, lysylprolylarginine, threonyllysylproline, threonyllysine, prolylarginine, lysylproline, choline, 4-(3-aminopropyl)-2-hydroxybenzoic acid and 4-(2-aminoethyl)-2-hydroxybenzoic acid.

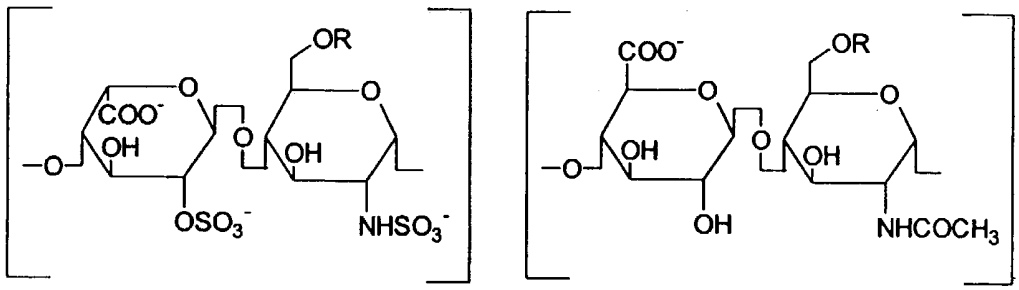
Patentansprüche

1. Verfahren zur Synthese der semisynthetischen Glykosaminoglykane der allgemeinen Formel III



III

worin p + q = m , wobei p eine andere Bedeutung als 0 besitzt und m und n ganze Zahlen bedeuten, deren Wert zwischen 1 und 100 liegt, R Wasserstoff oder den Sulfatrest (SO^-_3) bedeutet und $-Z(R_2)R_1$ eine nucleophile Gruppe bedeutet, worin Z Sauerstoff, Schwefel oder Stickstoff bedeutet, R_1 eine geradkettige oder verzweigtkettige (C_{1-12})-Alkylgruppe, Aminogruppe, eine aromatische Gruppe, eine Diazo- oder Hydroxylgruppe, substituiert oder nichtsubstituiert, bedeutet, R_2 nicht vorhanden ist oder eine geradkettige oder verzweigtkettige (C_{1-6})-Alkylgruppe bedeutet oder zusammen mit R_1 einen heterocyclischen Ring bildet, umfassend die Umsetzung eines Glykosaminoglykans mit Heparin- oder Heparanstruktur der allgemeinen Formel I



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mit 1 bis 200 Moläquivalenten, bezogen auf die dimere Einheit des Glykosaminoglykans mit Heparin- oder Heparanstruktur der allgemeinen Formel I, eines nucleophilen Reagenses, dessen Gruppe von der allgemeinen Formel II

$$R_1\text{-}Z\text{-}R_2 \quad \text{II}$$

mit umfaßt wird, in wäßriger Lösung, die eine Menge einer anorganischen oder organischen Base enthält, die ausreicht, irgendwelche in den nucleophilen Reagentien vorhandenen sauren Gruppen in Salzform zu überführen und/oder die gleichen nucleophilen Reagentien aus irgendwelchen Salzen, die sie mit Substanzen saurer Natur bilden, freizusetzen und einen Überschuß an Alkalität so zu ergeben, daß das Reaktionsgemisch von 0,5 bis 6 N ist, bezogen auf die verwendete Base, ggf. in Inertgasatmosphäre, unter Rühren während einer Zeit von 30 min und 24 h bei einer Temperatur zwischen 45°C und 95°C, Neutralisation des pHs der kalten wäßrigen Lösung durch Zugabe einer wäßrigen Lösung aus Chlorwasserstoffsäure, ggf. Entfernung des Überschusses an nucleophilem Reagens durch Extraktion mit einem mit Wasser nichtmischbaren Lösungsmittel oder durch Filtration, Unterwerfen der wäßrigen Lösung der Dialyse mit fließendem Wasser und destilliertem Wasser und Isolieren des Produkts mittels Lyophilisierung der wäßrigen Lösung, die es enthält, oder durch Präzipitation durch Zugabe eines geeigneten Lösungsmittels.

2. Verfahren nach Anspruch 1, dadurch **gekennzeichnet,** daß die Menge an nucleophilem Reagens zwischen 10 und 100 Moläquivalenten, bezogen auf die dimere Einheit des Glykosaminoglykans der allgemeinen Formel I, liegt und daß die Konzentration des Glykosaminoglykans in wäßriger Lösung zwischen 1 % und 5 % liegt.

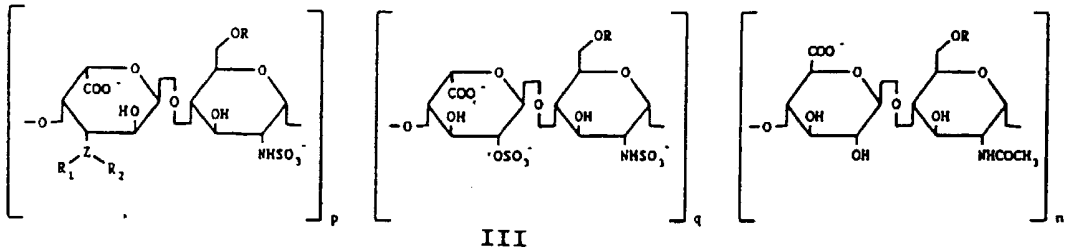
3. Verfahren nach Anspruch 1, dadurch **gekennzeichnet,** daß die verwendete Base ausgewählt wird aus Natriumhydroxid, Kaliumhydroxid und Triethylamin und daß der Überschuß an Alkalinität so ist, daß das Reaktionsgemisch von 1 N bis 3 N, bezogen auf die verwendete Base, ist.

4. Verfahren nach Anspruch 1, dadurch **gekennzeichnet,** daß sich die Gruppe $-Z(R_2)R_1$ von primären oder sekundären Aminen, sekundären heterocyclischen Aminen, Aminoalkoholen, Aminothiolen, Aminosäuren, Aminoestern, Peptiden, Alkoholen, Phenolen, Mercaptanen, Dithiolen, Thiophenolen, Hydroxylaminen, Hydrazinen, Hydraziden und Natriumazid ableitet.

5. Verfahren nach Anspruch 4, dadurch **gekennzeichnet,** daß sich die Gruppe $-Z(R_2)R_1$ von Glycin, Glycylglycin, L-Cystein, Acetyl-L-cystein, L-Cysteinethylester, 2-Aminothiophenol, 1,3-Propandithiol, Cysteamin, Natriumazid, 2-Aminoethylbisulfat, Taurin, Thioglykolsäure, β-Alaninethylester, L-Cystin, Hydroxylamin, Glycyltaurin, Cysteinyltaurin, Glycylcystein, Glycylphenylalanin, Glycyltyrosin, 2-Aminoethanol, Glycinester mit 2-Aminoethanol, Glycinamid mit 2-Aminoethanol, Arginyllysin, Arginin, Lysin, 2-Aminoethanolester mit Essigsäure, Salicylsäure, Methionin, Glycylprolin, γ-Aminobuttersäure, Lysylprolylarginin, Threonyllysylprolin, Threonyllysin, Prolylarginin, Lysylprolin, Cholin, 4-(3-Aminopropyl)-2-hydroxybenzoesäure und 4-(2-Aminoethyl)-2-hydroxybenzoesäure.

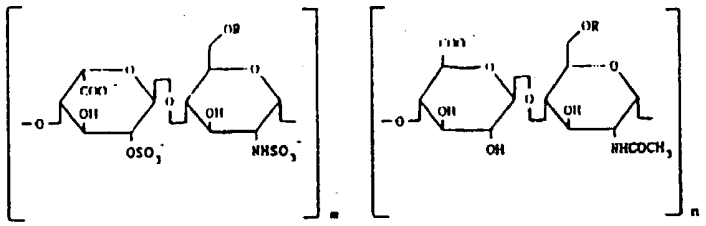
Revendications

1. Procédé de synthèse de glycosaminoglycanes de formule générale (III):



III

dans laquelle p + q = m , avec p non nul, et m et n sont des nombres entiers dont la valeur est comprise entre 1 et 100, R représente un atome d'hydrogène ou un résidu sulfate (SO_3^-) et $-Z(R_2)R_1$ représente un radical nucléophile dans lequel Z représente l'oxygène, le soufre, ou l'azote, R_1 représente des radicaux alkyle (C_{1-12}) linéaires ou ramifiés, amines, aromatiques, diazoïques, ou hydroxyle, substitués ou non substitués, et R_2 ne représente rien, ou représente un radical alkyle (C_{1-6}) linéaire ou ramifié, ou pris avec R_1, forme un cycle hétérocyclique, lequel procédé comprend la réaction d'un glycosaminoglycane avec une structure héparine ou héparane de formule générale (I):



A B

I

avec 1 à 200 équivalents molaires, par rapport à l'unité dimère du glycosaminoglycane avec la structure héparine ou héparane de formule générale (I), d'un réactif nucléophile dont le radical est inclus dans la formule générale (II):

$$R_1 - \overset{|}{Z} - R_2 \qquad (II)$$

dans une solution aqueuse contenant une quantité de base minérale ou organique suffisante pour salifier tous les groupes acide présents dans les réactifs nucléophiles, et/ou pour libérer les mêmes réactifs nucléophiles à partir de tous les sels qu'ils peuvent avoir avec des substances de nature acide, et pour générer un excès d'alcalinité tel que le mélange réactionnel est 0,5 à 6N par rapport à la base utilisée, éventuellement en atmosphère de gaz inerte, sous agitation pendant une période de temps comprise entre 30 minutes et 24 heures, à une température comprise entre 45°C et 95°C, l'ajustage à neutralité du pH de la solution aqueuse froide par addition d'une solution aqueuse d'acide chlorhydrique, éventuellement l'élimination de l'excès de réactif nucléophile par extraction à l'aide d'un solvant non miscible à l'eau, ou par filtration, la soumission de la solution à une dialyse avec de l'eau courante, et avec de l'eau distillée, et l'isolement du produit au moyen d'une lyophilisation de la solution aqueuse le contenant, ou par précipitation par addition d'un solvant approprié.

2. Procédé selon la revendication 1, caractérisé par le fait que la quantité de réactif nucléophile est comprise entre 10 et 100 équivalents molaires par rapport à l'unité dimère du glycosaminoglycane de formule générale (I), et que la concentration dudit glycosaminoglycane dans la solution aqueuse est comprise entre 1 % et 5 %.

3. Procédé selon la revendication 1, caractérisé par le fait que la base utilisée est choisie parmi l'hydroxyde de sodium, l'hydroxyde de potassium, et la triéthylamine, et que l'excès d'alcalinité est tel que le mélange réactionnel devient 1N à 3N par rapport à la base utilisée.

4. Procédé selon la revendication 1, caractérisé par le fait que le radical $-Z(R_2)R_1$ dérive d'amines primaires ou secondaires, d'amines hétérocycliques secondaires, d'aminoalcools, d'aminothiols, d'acides aminés, d'aminoesters, de peptides, d'alcools, de phénols, de mercaptans, de dithiols, de thiophénols, d'hydroxylamines, d'hydrazines, d'hydrazides, et d'azoture de sodium.

5. Procédé selon la revendication 4, caractérisé par le fait que le radical $-Z(R_2)R_1$ dérive de la glycine, de la glycylglycine, de la L-cystéine, de l'acétyl-L-cystéine, du L-cystéine éthyl ester, du 2-aminothiophénol, du 1,3-propanedithiol, de la cystéamine, de l'azoture de sodium, du 2-aminoéthyle bisulfate, de la taurine, de l'acide thioglycolique, du β-alanine éthyl ester, de la L-cystine, de l'hydroxylamine, de la glycyltaurine, de la cystéinyltaurine, de la glycylcystéine, de la glycylphénylalanine, de la glycyltyrosine, du 2-aminoéthanol, de l'ester de glycine avec le 2-aminoéthanol, de l'amide de la glycine avec le 2-aminoéthanol, de l'arginyllysine, de l'arginine, de la lysine, de l'ester du 2-aminoéthanol avec l'acide acétique, de l'acide salicylique, de la méthionine, de la glycylproline, de l'acide γ-aminobutyrique, de la lysylprolylarginine, de la thréonyllysylproline, de la thréonyllysine, de la prolylarginine, de la lysylproline, de la choline, de l'acide 4-(3-aminopropyl)-2-hydroxybenzoïque, et de l'acide 4-(2-aminoéthyl)-2-hydroxybenzoïque.